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Mucin glycoproteins are highly expressed by many tumors, reduce normal cell-cell and cell-extracellular matrix adhesion and protect cancer cells from attack by the immune system. Mucin expression not only increases, but also changes from a restricted pattern of apical expression to a general distribution over the entire cell surface. In this regard, conversion of prostate epithelium from a highly-organized, growth-controlled phenotype to a highly proliferative, metastatic phenotype is associated with loss of cell polarity. Very few studies been performed on mucin expression by prostate cancer cells. MUC1 is a large molecular weight, type I transmembrane mucin glycoprotein expressed by normal and malignant prostate epithelium. High level cell surface expression, reported immunosupressive activities of its released ectodomain, and antiadhesive properties all contribute to this mucin's ability to protect and promote tumor cell growth and survival. Recent observations using human breast cancer cell lines indicate that MUC1 can associate with the intracellular signal transducing molecules, β-catenin and GRB-2. Recent studies from the PI's lab demonstrate that cytokines, including interferon-γ, markedly stimulate MUC1 gene expression. Primary prostate tumors are often found in the vicinity of cytokine producing cells, and commonly metastasize to bone marrow, a rich source of these same cytokines.

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Introduction

MUC1 is a large, polymorphic mucin expressed on the surfaces of many normal and malignant epithelia (for review see references 1 and 2). Like other mucins, MUC1 is believed to provide protection to mucosal surfaces from both microbial and enzymatic attack as well as lubricate these cell surfaces. Over the last few years, it has become appreciated that MUC1 functions are more diverse. MUC1 is antiadhesive and inhibits cell-cell and cell-extracellular matrix interactions in both normal, e.g., embryo implantation, and pathological, e.g., cancer cells, contexts. In the case of cancer cells, MUC1 is often highly overexpressed and its antiadhesive properties may promote cell detachment from primary tumor sites as well as protection of the tumor cells from cell-mediated lysis. MUC1 expression is strongly regulated by steroid hormones in breast and uterine tissues *in vivo* and by proinflammatory cytokines *in vitro*.

The large externally-disposed portions of MUC1 (ectodomains) are released from normal and tumor cells where they can both absorb antibodies generated to tumor-specific MUC1 glycoforms as well as suppress immune cell function. Moreover, MUC1 has a transmembrane/cytoplasmic tail region that is highly conserved across species suggesting a conserved function. Recent studies indicate that the MUC1 cytoplasmic tail interacts with important signal transducing molecules, e.g., β-catenin and Grb2, and may participate in signal transduction events (3, 4). In spite of the number of studies of MUC1 expression and function in other systems, very little is known about MUC1 in the context of prostate cancer beyond that it is expressed in normal prostate epithelia and primary tumors. The proposed studies examine the impact of cytokines and androgens on MUC1 expression and function in androgen-sensitive and insensitive prostate cancer cell lines as well as normal prostate epithelia both at the level of gene expression and interactions with signal transducing proteins. A MUC1 gene knockdown approach will be used to disrupt MUC1 interactions with intracellular signal transducing molecules to determine the impact this has on prostate cancer cell growth.

Body

Our research accomplishments are detailed below, following the organization of the original proposal. MUC1 expression observed in most of the prostate cancer cell lines chosen for study has proven to be very low and not stimulated by either cytokines or testosterone. We extended these studies by examining MUC1 expression in tissue sections of primary prostate tumors provided by collaborators at Emory University. We have added studies of normal prostate epithelia to determine if regulation of MUC1 expression is fundamentally different between normal and transformed cells in this tissue.

Task I – To examine interferon-γ and androgen modulation of MUC1 gene expression

We have used both Western and Northern blotting approaches to examine MUC1 expression in primary cultures of normal human prostate epithelial cells (PrEC), LnCaP, C4-2, C4-2B, PC-3 as well as DU145 cell lines cultured with and without the presence of interferon-γ, TNF-α, dihydrotestosterone (DHT). PrEC cells displayed very little MUC1

by Western blotting; however, this expression could be stimulated by the addition of cytokines (interferon-γ plus TNFα [I+T]), but not DHT (Appendix ms. 1, Fig. 5; Appendix ms. 2, Fig. 1). Thus, normal prostatic epithelia require cytokine stimulation to produce MUC1. PC-3 cells responded similarly, except that interferon-γ alone was sufficient to drive MUC1 expression. In contrast, DU145 cells constitutively expressed MUC1 and this expression was not further stimulated by cytokines or DHT (Appendix ms. 2, Fig. 1). MUC1 was not detected in LnCaP, C4-2 or C4-2B cells under any condition (Appendix ms. 2, Fig. 1). In light of our preliminary observations that MUC1 was readily detectable by immunohistochemistry in normal prostate epithelia and primary tumors, we considered that loss of expression in the cell lines might be due to: 1) the *in vitro* culture conditions; 2) loss of MUC1 expression in metastic cells (all three cell lines were derived from metastases) or; 3) the requirement for combinations of cytokines and/or hormones for high level MUC1 expression in prostate cancer cells. We conducted additional studies of MUC1 expression in sections of over 300 primary prostate tumors in a human prostate tumor tissue microarray provided by collaborators at Emory University (Appendix ms. 2, Figs. 2-5, Tables 1 & 2). These studies revealed highly variable expression of MUC1. In some cases, strong staining consistent with cell surface expression was detected (left hand panels), while in other cases intracellular staining was evident (right hand panels); however, in most cases (57%) little or no staining was evident. Similar results were obtained using antibodies directed at either the ectodomain or cytoplasmic tail domains. In addition, in collaboration with Dr. Robert Sikes (Univ. of Delaware) we found that injection of either LnCaP or LnCaP-derived C42-B cells into mouse bone marrow failed to stimulate MUC1 expression in these cells (data not shown). Thus, even the complex mixture of factors present in bone marrow is insufficient to stimulate MUC1 expression in these androgen-responsive and non-responsive tumor cell lines. Expression of MUC1 did not correlate with tumor grade. These results were summarized and submitted for publication to *Prostate and Prostatic Disease.*

Task II – To define parameters by which interferon-γ or androgen modulate MUC1 association with β-catenin and GRB-2

These studies were originally planned to be initiated in months 16-22. In light of the inability to stimulate MUC1 expression in most primary prostate tumors and prostate cancer cell lines we abandoned these studies and focused our efforts on completing the analyses of the prostate tissue arrays discussed above.

Task III – To test effects of disruption of formation of MUC1 complexes with β-catenin and GRB-2 on prostate cancer cell growth in vitro

These studies were originally planned to be initiated in months 22-36. In light of the inability to stimulate MUC1 expression in most primary prostate tumors and prostate cancer cell lines we abandoned these studies and focused our efforts on completing the analyses of the prostate tissue arrays discussed above.

Key Research Accomplishments

1) Determination that neither interferon-γ, TNF-α nor DHT alone or in combination stimulates MUC1 expression in 4 out of 5 prostate cancer cell lines.
2) Determination that combined treatment of interferon-γ and TNF-α stimulates MUC1 expression in normal prostate epithelia and PC-3 cells.
3) Determination that MUC1 and MUC1SEC mRNA are expressed by cytokine-treated normal prostate epithelia (Appendix ms. 1, Fig. 5).
4) Determination that cell-associated MUC1 in normal prostate epithelia is in the form of an SDS-dissociable complex of the transmembrane/cytoplasmic tail with the ectodomain (Appendix ms. 1, Figs 1 and 4).
5) Determination that MUC1 is not highly expressed in a large fraction (>50%) of primary prostate tumors.
6) Determination that MUC1 expression is variable in normal and tumor-bearing prostate tissue and does not correlate with tumor grade.

Reportable Outcomes

This work has been published in two manuscripts.

Conclusions

It appears that neither normal prostate epithelia nor four out of five prostate cancer cell lines express high levels of MUC1 in their basal states. Normal prostate epithelia will greatly increase MUC1 expression when stimulated with combinations of cytokines (interferon-γ and TNF-α) shown to greatly stimulate MUC1 expression in other cellular contexts. These observations suggest that synergistic actions of cytokines are required to augment MUC1 production in prostate cancer cells as originally proposed. DHT has no impact on MUC1 expression in any scenario. Moreover, neither androgen-responsive or non-responsive prostate cancer cell lines express MUC1 when injected into the bone marrow of immunocompromised mice. Finally, we have found that while MUC1 is readily detectable in some primary prostate tumors, it is not in most. In light of these results, it does not appear to be fruitful to use MUC1 as a target of prostate cancer diagnosis or therapy.

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Appendices

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2. O'Connor, J.C., Julian, J., Lim, S.D. and Carson, D.D. MUC1 expression in human prostate cancer cell lines and primary tumors. Prostate Cancer and Prostatic Diseases, Submitted, 2004.

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Appendix Ms. 1

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Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells

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Abstract

MUC1 is abundantly expressed at the surface of epithelial cells in many tissues and their carcinomas. In humans, genetic polymorphism and differential splicing produce isoforms that may contribute to MUC1 participation in protection of the cell surface, modulation of cell–cell interactions, signaling, and metastasis. Biosynthetic and processing studies in tumor-derived cell lines indicate that cell surface MUC1 consists of a non-covalently associated heterodimer of separate cytoplasmic tail and extracellular domains. This heterodimer results from a single precursor proteolytically cleaved intracellularly. To determine whether similar processing of this isoform occurs in normal epithelial cells, we have examined cell-associated MUC1 and MUC1 released into medium by normal human uterine, mammary, and prostate epithelial cells. Cell-associated MUC1/REP was extracted as an SDS labile complex which was resistant to dissociation by boiling, urea, sulfhydryl reduction, peroxide, high salt, or low pH and was present in all normal cells examined. Addition of various proteolytic inhibitors during extraction had no effect on the proportion of this complex detected. MUC1 released into the medium was not associated with a particulate fraction (100,000*g* insoluble) and lacked the cytoplasmic tail. MUC1/REP and the MUC1 isoform lacking the transmembrane/cytoplasmic tail region, MUC1/SEC, mRNA were detected in all normal cells examined indicating that both shed and secreted MUC1 are likely to contribute to soluble forms found in culture media.

Keywords: MUC1; Mucin; Epithelia; Processing; Human; Uterus; Prostate

The extensively glycosylated ectodomains of transmembrane mucins protect the apical surface of normal glandular epithelium and generally create a surface nonadhesive to a variety of environmental pathogens and toxins as well as apposing epithelial cells [1–3]. Maintenance of this barrier function, as well as other functions attributed to transmembrane mucins, would be dependent upon regulation of mucin expression at the levels of transcription and translation, proper post-translational processing, and ultimately, regulation of ectodomain release. In normal cells, mechanisms by which these events are accomplished may be common for transmembrane mucins, regardless of species or tissue in which the individual mucins are expressed. Nonetheless, most studies of these events have utilized tumor derived or transformed cell lines in which altered transcriptional regulation [4–6], alternative splicing [7–9], aberrant glycosylation [10,11], and altered cellular distribution can occur [12,13]. Of all the transmembrane mucins identified so far, the most extensively examined is MUC1.

The polymorphic mucin MUC1 is expressed by most normal glandular epithelia and is found as both cell-associated and soluble forms (reviewed in [14,15]). Allelic variations in the number of tandem repeats in the extracellular domain of human MUC1 and alternative mRNA splicing contribute to the array of MUC1 forms detected in various systems. The predicted amino acid sequence describes a type I transmembrane protein possessing an extended extracellular domain composed mostly of 20 amino acid repeats varying in number with the individual alleles, a transmembrane domain, and a cytoplasmic tail of 69 amino acids [16–18]. Additional cell-associated forms, MUC1/Y [19,20] and MUC1/Z [21], identical to the full length molecule, but lacking the tandem repeats, have been detected in malignant cells.

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Soluble forms include the ectodomain of full length MUC1, termed MUC1/REP and a secreted form resulting from an alternative splicing event in which a stop codon is introduced into the sequence preceding the transmembrane and cytoplasmic domains, MUC1/SEC [18,22].

In certain tumor cells, full length MUC1 protein arrives at the cell surface as a heterodimeric complex, the result of an intracellular proteolytic cleavage of a single precursor protein shortly after translation in the endoplasmic reticulum [23]. The two cleavage products remain stably associated throughout intracellular processing and delivery to the cell surface. Although the association appears to be non-covalent, it survives multiple rounds of recycling from the cell surface to the Golgi for additional glycosylation and return to the surface [24]. Initial studies using recombinant MUC1 identified two potential cleavage sites within an 18 amino acid sequence located between 71 and 53 amino acid N-terminal to the transmembrane domain [23]. A subsequent study utilizing an epitope-tagged MUC1 has determined that one of the two Phe–Arg dipeptides is the intracellular cleavage site [25]. Furthermore, the cleavage event proved to be independent of host cell and uninfluenced by heavily O-glycosylated tandem repeat domains [25].

The soluble forms of MUC1 may be distinguished from each other by a unique amino acid sequence which lies at C-terminal on MUC1/SEC [22]. Both forms lack the transmembrane domain and cytoplasmic tail. While the tailless MUC1/SEC is clearly the product of alternative splicing, the more abundant form, MUC1/REP, is released from full length MUC1 under conditions in which alternative splicing could not occur [26]. The mechanism of release remains to be determined. Two possibilities have been suggested: dissociation of the complex formed as a result of metabolic processing [25] or a second cleavage (shedding) event [23]. Importantly, all of these studies have utilized cell lines derived from adenocarcinomas or transformed cell lines. Consequently, concern exists over whether formation of the heterodimeric MUC1 complex is a feature of tumor cells and not necessarily preserved in their normal counterparts. Therefore we have examined the processing of endogenous MUC1 in normal epithelial cells originating from several human tissues. These studies demonstrate that intracellular cleavage and formation of the heterodimeric complex are common to both normal and cancer cells and cell lines.

Materials and methods

Cell lines and culture conditions. The human uterine epithelial cell line, HES [27], was cultured in 24-well plates on matrigel (Collaborative Research) coated wells in high glucose Dulbecco's modified eagle's medium (Gibco BRL) supplemented with 10% (v/v) charcoal stripped fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM sodium pyruvate. Normal human mammary epithelial cells, HMEC, and normal human prostate epithelial cells, HPrE, were cultured on untreated 24-well plates in medium specified for each cell type by the supplier (Clonetics). MUC1 expression was induced by treatment with 200 U/ml interferon γ (IFγ) and 25 ng/ml tumor necrosis factor α (TNFα) for 48 h. The human mammary carcinoma cell line, T47D, was obtained from the American Tissue Culture Collection and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of cell lysates. Unless otherwise specified, cell lysates were prepared from confluent cultures by addition of 0.5% (v/v) Nonidet P-40 in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetracetic acid and a protease inhibitor mixture at a final dilution of 1:100 (v/v). The protease inhibitor mixture was composed of 10 mg/ml chymostatin, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 10 mg/ml benzamidine, 2 mg/ml antipain, and 7.6 trypsin-inhibiting U/ml aprotinin in 0.9% (w/v) NaCl and 0.9% (v/v) benzyl alcohol. Lysis buffer was added at 98 °C. After 5 min at room temperature, the cell residue was scraped into the lysis buffer and the lysate was centrifuged for 15 min at 4 °C at 10,000*g*.

Treatment of cell lysates. Cell lysates were exposed to 6 M urea, 5% (v/v) β-mercaptoethanol, or 1% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 30 min. Urea or β-mercaptoethanol was removed by passing the samples over dextran desalting columns (Pierce) equilibrated with 0.5% (v/v) Nonidet P-40 (NP-40) in PBS. The sample containing SDS was diluted to 0.1% (w/v) SDS with 0.5% (v/v) NP-40 in PBS. To determine the effect of low pH on the MUC1 metabolic complex, lysate was brought to pH 5.0 by the addition of HCl, incubated for 1.5 h at room temperature, and brought back to pH 7.4 by addition of NaOH. To determine the effect of high salt, lysate was made 2 M NaCl by the addition of 4 M NaCl in PBS, incubated for 1.5 h at room temperature, and diluted to 0.15 M NaCl with 0.5% (v/v) NP-40. To test the effect of elevated temperature, lysate was boiled for 10 min and chilled to room temperature. Lysate was incubated for 2 h with 100 μM hydrogen peroxide at room temperature, followed by removal of peroxide by passage over a dextran desalting column. Metalloprotease inhibitor Illomostat (GM6001, Chemicon) was dissolved in dimethyl sulfoxide and added to lysis buffer at a concentration of 10 μg/ml. Furin inhibitor ([28]; CalBiochem) was dissolved in methanol and added to cell culture medium and lysis buffer at a concentration of 25 μM. After all treatments, the lysate was divided equally prior to immunoprecipitation.

Preparation of total cell extracts and released protein. For preparation of total cell extracts, confluent cultures were solubilized by addition of 0.05 M Tris, pH 7.0, 8 M urea, 1.0% (w/v) SDS, 1.0% (v/v) β-mercaptoethanol, and 0.01% (w/v) phenylmethylsulfonyl fluoride at room temperature. For collection of released/secreted proteins, cultures were rinsed once with serum-free medium and cultured for 24 h in serum-free medium. Conditioned medium was centrifuged at 4 °C for 10 min at 10,000*g* and used directly for immunoprecipitation or concentrated by precipitation at 4 °C with 10% (w/v) trichloracetic acid (2 μl fetal bovine serum was added per 250 μl medium as carrier). Precipitates were rinsed once with acetone, air dried, and dissolved in equal volumes of sample extraction buffer (above) and Laemmli's sample buffer [29] containing 20% (v/v) glycerol. Total cell extracts were precipitated as described, without carrier.

Vectorial biotinylation of MUC1 at the cell surface. Confluent cultures of HES were aseptically rinsed with ice-cold PBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (PBS-CM) and incubated at 4 °C for 30 min in the dark with 10 mM sodium metaperiodate in 0.1 M sodium acetate, pH 5.0. Periodate oxidation was terminated by addition of cold PBS-CM containing 200 mM sucrose for 5 min followed by two

rinses with Hank's balanced salt solution (HBSS). Cells were then incubated at 4 °C for 2 h in the dark with 100 μg/ml biotin hydrazide (Pierce) in HBSS. The reaction was terminated by rinsing three times with ice-cold HBSS. Prewarmed serum-free medium was added and the cultures were processed immediately or returned to the incubator for 24 h at 37 °C. Control cultures were rinsed with cold PBS and maintained at 4 °C in PBS for the same period of time but were not subjected to the biotinylation protocol. In this protocol, the O-linked glycans are biotinylated rather than the protein core.

Immunoprecipitation and Western blot analysis. Lysate (125 μl) or medium (500 μl), representing one half of sample obtained from a confluent well, was incubated by constant rotary agitation overnight at 4 °C with rabbit polyclonal antibody CT-1 [30,31] at the ratio of 1:60, serum to medium or 1:15, serum to lysate. Mouse monoclonal antibody 214D4 [32] was added at the ratio of 1:25, hybridoma medium to medium or 1:6, hybridoma medium to lysate. Antigen–antibody complexes were incubated for 8 h at 4 °C with constant agitation after addition of 50 μl of a 50% (v/v) slurry of protein G–Sepharose (Sigma) that had been previously blocked with fetal bovine serum. The pelleted resin was washed twice with 500 μl of 0.5% (v/v) NP-40 in PBS and twice with PBS. Resin pellets were extracted by boiling 2 min in 50 μl sample extraction buffer (above) and 50 μl Laemmli's sample buffer, followed by centrifugation. Samples (25 μl) were separated on 10% (w/v) SDS–polyacrylamide gels [33], transferred to nitrocellulose in 100 mM Tris base, 100 mM glycine, pH 8.3, and blocked for 4 h at 4 °C in 3% (w/v) BSA (fraction V, Sigma), 0.1% (v/v) Tween 20 in PBS. Primary antibody 214D4 was used at a dilution of 1:10,000 in blocking buffer. After an overnight incubation at 4 °C, the blots were rinsed three times for 5 min in 0.5% (v/v) Tween 20 in PBS. Secondary antibody, HRP-conjugated donkey anti-mouse IgG (Jackson Immunologicals), diluted 1:200,000 in blocking buffer was added for 2 h at 4 °C. Blots were rinsed three times 5 min in 0.5% Tween 20 in PBS. Antibody was visualized by ECL reagents applied according to manufacturer's directions (Pierce).

RNA isolation and RT-PCR analysis. Total RNA was isolated from confluent cultures using an RNeasy kit and QIA-shredder columns according to manufacturer's directions (Qiagen). Genomic sequences were removed by deoxyribonuclease treatment (DNA-free Kit, Ambion) and a mock treated sample of each was performed in parallel. Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl reaction mixture using random hexamers and kit components at the recommended concentrations (Gene Amp RNA PCR Kit, Applied Biosystems). The reaction mixture was maintained at room temperature for 10 min, 15 min at 42 °C, 5 min at 99 °C, and 5 min at 4 °C. A negative control lacking template was included in each reverse transcription and subsequent amplification experiment. In addition, a duplicate sample containing template but lacking reverse transcriptase was included. Samples for PCR were prepared by addition of 10 μl of RT reaction to 40 μl of a PCR mixture containing kit components at the recommended concentrations and primers (0.2 μM each). Primers for MUC1/REP corresponded to bases 729–748 (forward primer: TGCATCAGGCTCAGCTTCTA) and bases 1257–1276 (reverse primer: GAAATGGCACATCACTCACG); product size was 548 bp. Primers for MUC1/SEC corresponded to bases 729–748 (forward primer: TGCATCAGGCTCAGCTTCTA) and bases 1068–1087 (reverse primer: GGAAGGAAAGGCCGATACTC); product size was 359 bp. Primers for human ribosomal protein L19 were: forward primer-CTGAAGGTGAGGGGAATGTG and reverse primer-GGATAAAGTCTTGATGATCTC; product size was 239 bp. After an initial incubation for 2 min at 95 °C, samples were amplified for 35 cycles of 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C for MUC1/REP and MUC1/SEC or 25 cycles of 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C for L19 with a final period of 5 min at 72 °C. PCR products were resolved on 1.2 % (w/v) agarose gels in 40 mM Tris acetate 2 mM ethylenediaminetetraacetic acid, pH 8.5.

Results and discussion

Cell-associated MUC1 exists as an SDS-labile complex in normal epithelial cells

Using the human breast cancer cell line T47D, one of the cell lines in which the intitial obervations on the metabolic cleavage and complex formation of MUC1 were made [23], we first confirmed the nature of the MUC1 complex and examined conditions that might affect its formation and/or dissociation. In parallel, the cell line HES, a cell line derived from normal proliferative phase human uterine epithelium [27], was examined. Cell-associated MUC1 was extracted with NP-40 in the presence or absence of β-mercaptoethanol, urea or SDS. If the complex remained intact, all conditions would yield similar amounts of MUC1 immunoprecipitated with the cytoplasmic tail-directed antibody (CT-1). If, however, the complex was disrupted by one or more conditions, this would be reflected in the diminished ectodomain signal when immunoprecipitated with antibody recognizing the cytoplasmic tail. As was the case for cell-associated MUC1 extracted from ZR-75-1 breast carcinoma cells [23], MUC1 extracted from HES could be dissociated by exposure to SDS, but not urea or β-mercaptoethanol (Fig. 1a). Exposure of cell-associated MUC1 to high salt, low pH, or peroxide also failed to dissociate the complex (Figs. 1b and c). Cell-associated MUC1 extracted from T47D breast cancer cells displayed a similar sensitivity to SDS (data not shown). Utilizing the susceptibility of the complex to SDS disruption, two other normal epithelial cells were examined for the presence of MUC1 metabolic complex. In this case, the cells were induced to produce sufficient MUC1 for analysis by combined treatment with interferon γ and TNFα (E. Lagow and D.D. Carson, manuscript submitted). Cell-associated MUC1 in both normal human mammary epithelial cells (HMEC) and normal human prostate epithelial cells (HPrEs) exhibited a similar susceptibility to SDS (Fig. 1d). Thus, cell-associated MUC1 in normal cells appears to undergo a similar metabolic cleavage event and form an SDS-dissociable complex as reported for tumor derived cell lines.

The use of SDS disruption to demonstrate the presence of the metabolic complex raised the question of what portion of endogenous full length MUC1 was subjected to cleavage. In some early experiments, exposure to SDS led to a reduction, but not complete disappearance of detectable MUC1 ectodomain precipitable by CT1. This could have resulted either from a partial cleavage of MUC1 during metabolic processing or an incomplete dissociation of the preformed complex during exposure to SDS. Extending the period of exposure to SDS to 1 h produced a uniform complete disruption of the metabolic complex as determined by

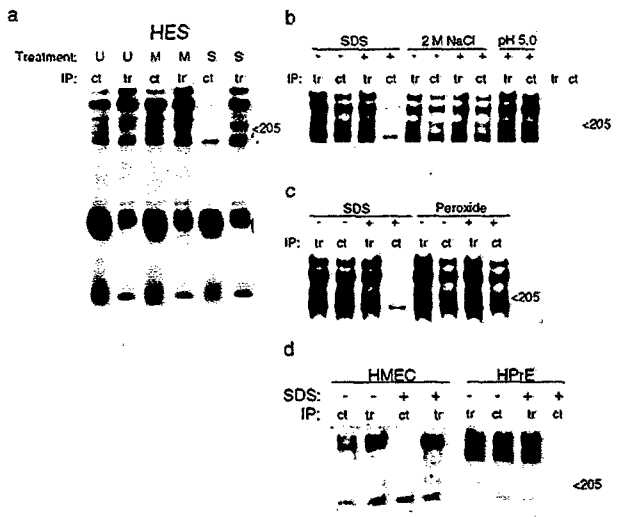

Fig. 1. Cell-associated MUC1 expressed by normal human epithelial cells exists as an SDS labile complex. (a) Prior to immunoprecipitation, lysates of HES were treated with either 6 M urea (U), 5% (v/v) β-mercaptoethanol (M), or 1% (w/v) SDS (S). Treated lysates were split equally and immunoprecipitated with either anti-C terminal peptide, CT-1 (ct), or anti-tandem repeat, 214D4 (tr). The Western blot was probed by antibody 214D4. Bands detected below 205 kDa are due to components of the IgG used for immunoprecipitation (*). (b) Prior to immunoprecipitation, lysates of HES were treated with either 1% (w/v) SDS, 2 M NaCl, or pH 5. Treated lysates were split equally and immunoprecipitated as described in panel A. The last two lanes contain no lysate, only antibody. The Western blot was probed by antibody 214D4. (c) Prior to immunoprecipitation, lysates of HES were treated with either 1% (w/v) SDS or 100 μM hydrogen peroxide. Treated lysates were split and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4. (d) Prior to immunoprecipitation, lysates of HMEC or HPrE were treated with (+) or without (−) 1% (w/v) SDS. Treated lysates were split equally and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4.

the lack of detectable MUC1 ectodomain precipitable by CT-1 and this condition was used in all subsequent experiments. This result agrees with the observation of Parry et al. [25] that almost no uncleaved MUC1 could be detected in whole cell lysates of several different tumor cell lines originating from various tissues. Therefore, metabolic cleavage and heterocomplex formation appear to be an intrinsic feature of full length MUC1 processing in normal epithelial cells as well and is independent of the tissue source.

The extended processing time involved in extraction, SDS exposure, and immunoprecipitation of samples led us to consider the possibility that a portion of the "metabolic cleavage" may occur after disruption of the cell and exposure of MUC1 to solubilized proteases that would not otherwise have access to MUC1 during its transit through or residence at the surface of the intact cell. Two lines of evidence provided in the studies of others indicate that this is probably not the case. MUC1 translated in vitro or within a cell context and extracted under similar conditions demonstrated a progressive degree of cleavage dependent upon the period of metabolic processing [23]. Although these experiments were performed with a truncated form of MUC1 containing only one tandem repeat, it has been determined in a subsequent study that neither the number of tandem repeats nor the degree of glycosylation of the tandem repeats affects the site or extent of metabolic cleavage [25]. To exclude the possibility of post-extraction cleavage, we evaluated the effect of various conditions of extraction on the proportion of complex detected. Cells were extracted by the addition of boiling 0.5% Nonidet P40 containing 1 mM EDTA, and a cocktail of serine protease inhibitors for 5 min, 0.5% Nonidet P40 containing no inhibitors at 4 °C for 1 h or 0.5% Nonidet P40 containing 1 mM EDTA and a cocktail of serine protease inhibitors at 4 °C for 1 h. Neither elevated temperature nor inclusion of a proteolytic inhibitor cocktail reduced the subsequent proportion of SDS labile complex in the resulting lysate (data not shown). Inclusion of a broad spectrum metalloproteinase inhibitor, Illomostat [34], in addition to the protection afforded by 1 mM EDTA, did not affect the proportion of SDS-labile complex present in the resulting lysates (Fig. 2a). Pretreatment during culture and inclusion of a furin inhibitor during lysis also did not affect the proportion of SDS-labile complex (Fig. 2b). Contrary to the results of Ligtenberg et al. [23], we could find no evidence that exposure to elevated temperature resulted in the dissociation of the metabolic complex. Neither lysis at elevated temperature nor boiling the lysate after extraction (Fig. 2a) resulted in dissociation of the complex unless SDS was present.

Normal epithelial cells release a tailless, soluble form of MUC1

The ultimate fate of the MUC1 metabolic complex upon arrival at the cell surface remained to be determined in normal cell lines. Release of the ectodomain as a soluble form rather than lysosomal degradation appears to be the fate of the majority of MUC1 arriving at the surface of tumor cells. In tumor cell lines all surface-associated MUC1 molecules were constitutively internalized and recycled to the surface until release [24]. Transport to the lysosomes was considered to be of minor importance. As noted earlier, the two soluble forms of MUC1 released by tumor cell lines lack the cytoplasmic tail. We confirmed that the forms of MUC1 released into the medium by normal human epithelial cells were not membrane-associated (Fig. 3) and lacked the cytoplasmic domain (Fig. 4). These experiments further demonstrated that the MUC1 released to the medium from normal cells could not be the product of cell lysis which would release membrane fragments containing MUC1 metabolic complex. If this were the

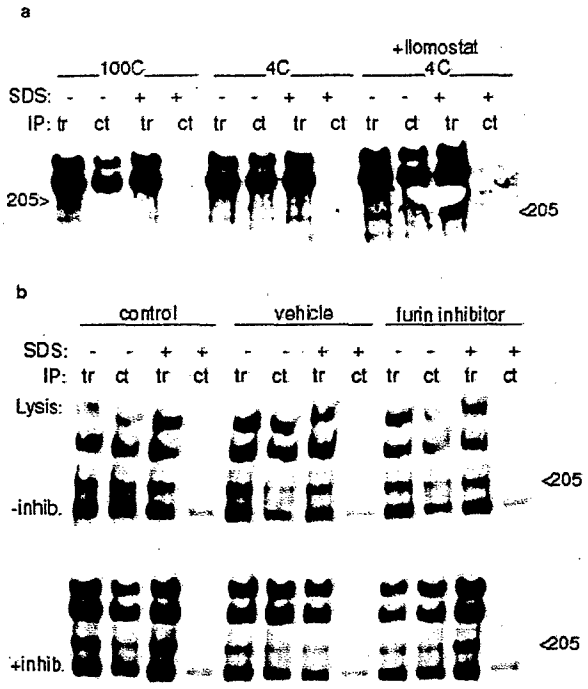

Fig. 2. MUC1 metabolic complex expressed by normal human epithelial cells does not dissociate at 100 °C and is not further proteolytically cleaved during lysis. (a) HES were lysed in the standard lysis buffer with or without 10 μg/ml Illomostat for 1 h at 4 °C or lysis buffer was added at 98 °C, followed by 5 min at room temperature and 10 min at 100 °C before centrifugation. The treated lysates were split equally and immunoprecipitated with anti-C terminal peptide, CT-1 (ct) or anti-tandem repeat, 214D4 (tr). The Western blot was probed by antibody 214D4. (b) HES cultures were incubated for 24 h with no addition (control), vehicle, or 25 μM furin inhibitor prior to extraction. Lysates were prepared in standard lysis buffer containing vehicle (upper set) or 25 μM furin inhibitor (lower set). Lysates were split equally and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4.

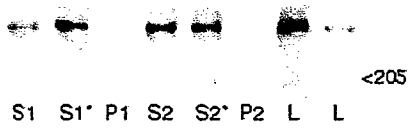

Fig. 3. MUC1 released into medium by HES is not associated with particulates. Serum free medium conditioned by HES for 24 h was centrifuged for 10 min at 10,000*g*. Two 2 ml samples were further centrifuged for 1 h at 100,000*g*. The upper 1 ml of each supernate (S1, S2) and the lower 1 ml (S1*, S2*) were removed and the bottom of the tubes were extracted with sample extraction buffer (P1, P2). All were TCA precipitated as described in Materials and methods. A 1 ml and 0.5 ml sample not subjected to 100,000*g* centrifugation (L) were included for comparison. Precipitates were resolved by SDS–PAGE and the Western blot was probed by antibody 214D4.

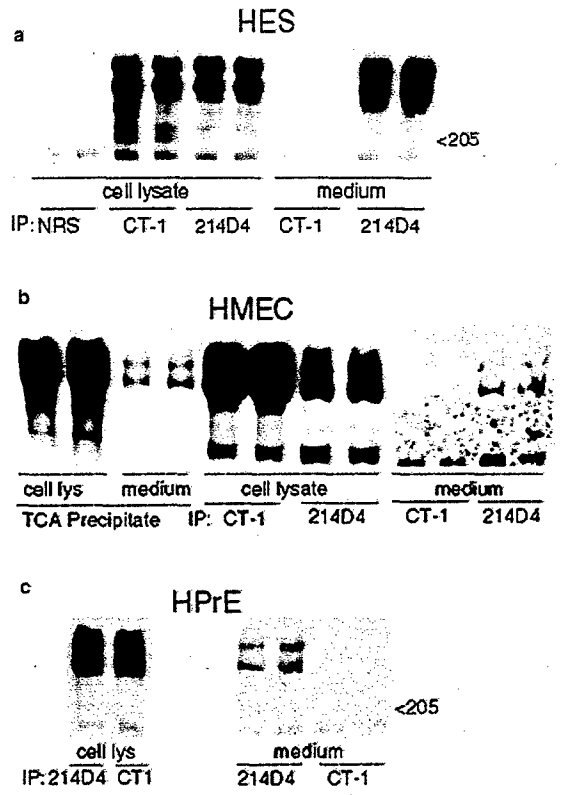

Fig. 4. MUC1 released into medium by normal human epithelial cells lacks the cytoplasmic tail. (a) Serum free medium conditioned by HES for 24 h or cell lysate was immunoprecipitated with normal rabbit serum (NRS), anti-C terminal peptide (CT-1) or anti-tandem repeat (214D4) in duplicate. The Western blot was probed by antibody 214D4. (b) Serum free medium conditioned by HMEC for 48 h or cell lysate was immunoprecipitated as in panel A or TCA precipitated in duplicate as described in Materials and methods. The Western blot was probed by antibody 214D4. (c) Serum free medium conditioned by HPrE for 48 h or cell lysate was immunoprecipitated as in panel A. The Western blot was probed by antibody 214D4.

case then CT-1 antibody would have immunoprecipitated the MUC1 ectodomain. However, these experiments do not distinguish between the two possible soluble forms of MUC1. Although production of the splice variant MUC1/SEC has been detected in secretions of normal fallopian tube and uterus of a lower primate [35], its production by normal human cells has not been examined. If MUC1/SEC could not be detected in normal human cells, the threefold possibilities for the origin of the tailless MUC1 ectodomain in secretions from a variety of normal human tissues would be reduced to two: it is either generated from the dissociation of the MUC1 metabolic complex or it results from an additional proteolytic cleavage step. However, mRNA for MUC1/SEC was detected by RT-PCR analysis of total RNA extracted from HES, HMEC, or HPrE (Fig. 5). Thus, a portion of soluble MUC1 released by these cells could be due to secretion of MUC1/SEC. Nonetheless, the majority of soluble MUC1 must arise from the predominant form of MUC1 expressed by normal cells, MUC1/REP. Under conditions in which alternative splicing was not possible, transformed mouse mammary cells transfected with full length human

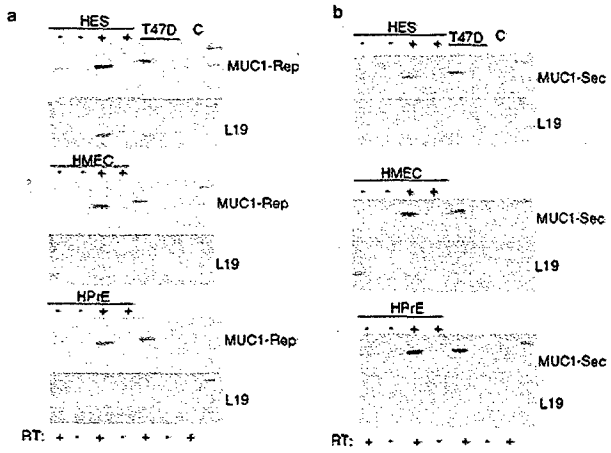

Fig. 5. Normal human epithelial cells express mRNA for both MUC1/REP and MUC1/SEC. Total RNA extracted from HES, HMEC, HPrE, or T47D was analyzed by RT-PCR for the expression of mRNA encoding MUC1/REP (panel a), MUC1/SEC (panel b), and ribosomal protein L19. HES were either cultured in medium containing 10% (v/v) serum (+) or withdrawn from serum for 48 h (–). As indicated at the top of each series, HMEC and HPrE were cultured either with (+) or without (–) interferon γ and TNFα for 48 h as described in "Materials and methods." As indicated at the bottom of each series, reverse transcription (RT) was performed on 1 μg total RNA in the presence (+) or absence (–) of reverse transcriptase. Control (C) contained no template.

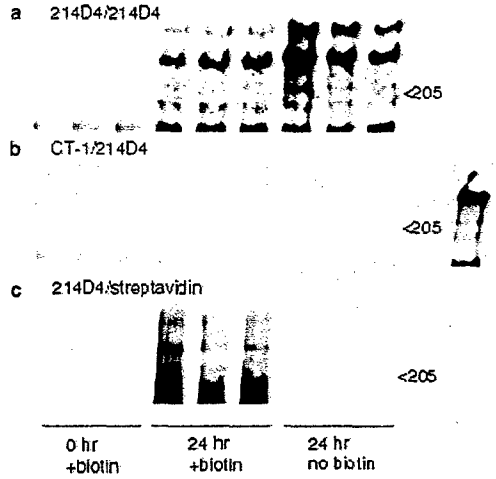

Fig. 6. MUC1 vectorially biotinylated at the cell surface is released into the medium lacking the cytoplasmic tail. HES were biotinylated at the cell surface as described in "Materials and methods." Control cultures were rinsed and maintained at 4 °C, but were not biotinylated. Serum free medium was added and collected immediately after biotinylation (0 h) or after 24 h incubation at 37 °C. Equal amounts of medium were immunoprecipitated with anti-tandem repeat (214D4; panels a and c) or anti-C terminal peptide (CT-1; panel b). Western blots of triplicate individual samples were probed by antibody 214D4 (panels a and b) or HRP-streptavidin (panel c). The immunoprecipitating antibody/Western blot probe is indicated above each panel. The sample to the right of the middle panel is the last control from the top panel included as a positive control for the Western blot.

MUC1 released a tailless form of MUC1 into the medium [26]. As demonstrated in Fig. 6, MUC1 biotinylated at the cell surface appears as a tailless form released to the medium within 24 h. During the same time period, cell-associated, biotinylated MUC1, precipitable by both CT-1 and antibody 214D4, disappeared (data not shown). MUC1/SEC would not be expected to be retained sufficiently long at the cell surface to become biotinylated nor would it be immunoprecipitated by the CT-1 antibody.

The remaining question is the pathway of release: dissociation of the metabolic complex or an additional cleavage step. A simple dissociation is unlikely for a number of reasons, first of which is the apparent stability of the metabolic complex. The complex must remain associated not only through the steps of metabolic processing from the endoplasmic reticulum to delivery at the cell surface, but it must also be sustained through multiple rounds of recycling from the cell surface to the golgi for additional glycosylation (sialylation) with return to the surface [24]. The results of the present study further demonstrate the stability of the complex when exposed to conditions that might be encountered at the surface or during recycling. Secondly, the failure to detect an additional cleavage fragment, i.e., an N-terminus distinct from that of the metabolic cleavage site [25], does not exclude the possibility of a second cleavage event. Those studies were performed on immunoprecipitated cell-associated metabolic complex. If a second cleavage event had occurred, the residual membrane-associated fragment with a potentially different amino terminus would not have been included in the immunoprecipitates produced by the antibody to the FLAG sequence incorporated into the ectodomain. Finally, if no complex was formed, no release of ectodomain would be expected if the mechanism was simple dissociation; however, cells transfected with a mutant form of full length MUC1 lacking the metabolic cleavage site were reported to release as much MUC1 as those expressing the wild type form [23]. Since no metabolic complex was formed, release of the ectodomain had to have occurred by another mechanism.

These results indicate that processing of the full length MUC1 core protein is similar in both normal and tumor cells, regardless of differences in glycosylation or the production of isoforms. The identity of the protease responsible for the metabolic cleavage, thought to be a member of the kallikrein family [23,25], remains to be determined, as does the mechanism by which the ectodomain is ultimately released from the metabolic complex. Comparison of amino acid sequences suggests that the metabolic cleavage of MUC1 is conserved across species [25]. Cleavage and formation of a metabolic complex as an initial step of metabolic processing are predicted to occur for MUC3 and MUC12 [25] based on sequence homologies to the MUC1 cleavage

site, and is a feature of MUC4 metabolism [36]. Although the cleavage site sequence in MUC4 differs from that of the other transmembrane mucins, it is also conserved across species and complex formation occurs in both normal tissues and tumor cells [5]. However, metabolic cleavage may not prove to be a universal event in transmembrane mucin processing. Epiglycanin, a large transmembrane murine mucin for which the human homolog is yet to be identified, does not form a metabolic complex [37].

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Appendix MS.2

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MUC1 expression in human prostate cancer cell lines and primary tumors

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MUC1 expression was evaluated in normal prostate epithelial cells (PrEC), and prostate cancer cell lines in response to dihydrotestosterone (DHT), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) treatment. Expression of MUC1 core protein was stimulated in PrEC and PC-3 cells after cytokine treatment, but was highly and constitutively expressed by DU-145 cells. MUC1 was not expressed by LNCaP, C4-2 or C4-2B cells under any condition. DHT alone or in combination with cytokines had no effect on MUC1 expression in any cell line tested. Using antibodies capable of detecting all isoforms of MUC1 core protein independent of their glycosylation state, immunohistochemical staining of tissue microarrays containing both nontumor and tumor tissue revealed that only 17% of tumor tissues and 41% of nontumor tissues stained positively for MUC1. Staining patterns in tumor tissue varied from focal apical staining to diffuse cytoplasmic staining. Neither the presence of MUC1 core protein nor its subcellular distribution correlated with Gleason grade. These data indicate that MUC1 is a poor marker of prostate cancer progression. Furthermore, IFN-γ and TNF-α strongly induce MUC1 expression in both normal prostate epithelia and certain prostate tumor cell lines and may exacerbate pathologies associated with MUC1-positive prostate cancers.
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Keywords: MUC1; mucin; prostate cancer

Introduction

Mucins are high molecular weight glycoproteins that are found almost exclusively on the apical surface of many glandular epithelia including the gastrointestinal, respiratory, urinary, and reproductive tracts (reviewed in Gendler,[1] Gendler and Spicer,[2] Lagow *et al*,[3] Taylor-Papadimitriou *et al*[4]). MUC1 is a type I transmembrane glycoprotein, consisting of a very large (1000–2200 amino acids) extracellular domain comprised of a series of 20–25 amino-acid tandem repeats, a 31-amino-acid transmembrane domain, and a short (72 amino acids) cytoplasmic tail. MUC1 primarily functions in lubrication and hydration of epithelia and protection from microbial attack. However, the large extended extracellular domain of MUC1 also appears to play both adhesive and antiadhesive functions and contributes to decreased immune response. More recently, MUC1 has been implicated in signal transduction due to interactions of its highly conserved cytoplasmic tail with several signal transducing molecules[5–7] (reviewed in Gendler[1]).

Aside from its normal physiological roles, MUC1 also has been implicated in progression of numerous types of cancer including breast, colon, lung, gastric, and pancreatic cancers (reviewed in Gendler and Spicer,[2] Taylor-Papadimitriou *et al*,[4] Ho *et al*[8]). MUC1 expression in tumors is greatly increased and accompanied by altered glycosylation and aberrant expression patterns that become more diffuse when compared to the normal apically restricted pattern.[9–12] Moreover, MUC1 is proposed to help tumor cells evade host defenses by attenuating immune responses and to promote metastasis through a loss of cell–cell and cell–extracellular matrix (ECM) contact (reviewed in Taylor-Papadimitriou *et al*[4]).

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While the role of MUC1 in the progression and metastasis of several common cancers is well documented, the role of MUC1 in prostate cancer has received relatively little attention. There is considerable variability in MUC1 expression reported in normal prostate epithelia, from strongly positive immunoreactivity[8] to weak and/or no immunoreactivity.[8,9,12] In each of these cases, however, the apically restricted expression pattern is consistent with other nonneoplastic epithelia. The few studies that are available suggest that MUC1 also plays a role in progression and metastasis of prostate cancer, usually with higher immunoreactivity, altered cell-surface expression patterns (from apical expression to diffuse staining), and altered glycosylation, although there is still considerable variability among studies.[8,9,12–17] Unfortunately, most of these reports are limited by small sample sizes and are confounded by differences in methodology (eg, antibodies used, processing of tissue samples) and the complexity of the disease itself.

In the current report, expression of MUC1 core protein has been evaluated in primary cultures of normal prostate epithelia and several commonly used prostate cancer cell lines under various treatment conditions. In addition, a combination of antibodies capable of detecting all forms of MUC1 independent of glycosylation state was used to examine MUC1 expression in tissue microarrays containing 278 tissue sections taken from 110 patients. MUC1 expression and subcellular distribution in tumor cells were correlated with Gleason grade. This represents the largest single study yet undertaken to examine MUC1 expression in human prostate cancer.

Materials and methods

Cell culture

Five human prostate carcinoma cell lines and primary cultures of normal prostate epithelia were used. LNCaP, PC-3, and DU-145 human prostate carcinoma cell lines (American Type Culture Collection (ATCC), Manassas, VA, USA) and C4-2 and C4-2B$_4$ (obtained from Dr Leland Chung, Emory University School of Medicine, Atlanta, GA, USA) were maintained in RPMI-1640 (catalog #11875; Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 μg/ml streptomycin. Normal human prostate epithelial cells (PrEC; Clonetics, San Diego, CA, USA) were maintained in the manufacturer's recommended growth medium (Clonetics™ Prostate Epithelial Cell Medium). All cultures were maintained at 37°C in a humidified air: CO_2 (95 : 5; v/v) atmosphere. Cells were plated onto six-well culture plates at a density of 500 000 cells/well and maintained until approximately 90% confluent. Cells then were cultured for 24 h in phenol red-free RPMI-1640 media without FBS prior to treatment with phosphate-buffered saline (PBS; control), 100 ng/ml tumor necrosis factor alpha (TNF-α; Roche Biochemicals, Indianapolis, IN, USA), 50 ng/ml interferon gamma (IFN-γ; Roche Biochemicals), 1 μM dihydrotestosterone (DHT), or combined treatments with both cytokines and DHT prepared in phenol red-free RPMI-1640 media without FBS. Combined treatment with these cytokines has synergistic effects on MUC1 expression in T47D human breast ductal carcinoma cells.[18] The concentrations used in the current report were selected based on these previous studies and other published reports and represents the highest effective concentration common to these studies. It was expected that if cytokines or DHT were stimulatory for MUC1 expression then the effect would be observed at these concentrations. DHT was dissolved in ethanol (0.1% v/v final concentration). All treatments with cytokines and DHT were for 24 h prior to sampling.

Western blot analysis

Total cellular protein was prepared by solubilizing the adherent cells with 500 μl of sample extraction buffer (SEB; 50 mM Tris-HCl pH 7.0, 8 M urea, 1% (v/v) sodium dodecyl sulfate (SDS), 1% (v/v) 2-mercaptoethanol, 0.01% (w/v) phenylmethylsulfonylfluoride (PMSF)). Protein concentration was determined after trichloroacetic acid (TCA) precipitation by the method of Lowry *et al*.[19] For PC-3 and DU-145 cells, 5 μg of total cell protein was analyzed for MUC1; for PrEC, LNCaP, C4-2, and C4-2B cells, 40 μg of total cell protein was analyzed for MUC1. Total cellular protein (5 μg) from HES cells, a human uterine epithelial cell line[20] was analyzed concurrently as a positive control.[21] Protein was concentrated by TCA precipitation overnight at 4°C. The resulting pellets were rinsed with acetone, air-dried for 10 min at room temperature, redissolved in 20 μl SEB and 20 μl Laemmli sample buffer (LSB; Biorad, Hercules, CA, USA), and heated at 95°C for 2 min prior to loading.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% (w/v) polyacrylamide stacking gel[22] and a 10% (w/v) polyacrylamide resolving gel[23] in gel running buffer (150 mM glycine, 50 mM tris-base, 0.1% (v/v) SDS, pH 8.8) under constant voltage. Proteins were transferred to a nitrocellulose membrane at 4°C for 5 h at 40 V in transfer buffer (100 mM glycine, 100 mM tris-base, pH 8.3). The nitrocellulose membranes were blocked overnight at 4°C with gentle rotary agitation in PBS containing 0.1% (v/v) Tween-20 (PBS-T) and 3% (w/v) bovine serum albumin (BSA). Following the blocking step, the MUC1 214D4 primary antibody (kindly provided by Dr John Hilkens of the Netherlands Cancer Institute, Amsterdam, The Netherlands), a mouse monoclonal antibody specific for a tandem repeat epitope in the extracellular domain of MUC1,[24] was added to blocking solution at a 1 : 10 000 dilution and incubated overnight at 4°C with gentle rotary agitation. The blots were washed three times for 10 min each at room temperature with PBS-T. The secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA), was added to blocking solution at a 1 : 200 000 dilution and incubated for 2 h at 4°C with gentle rotary agitation. After three 10 min washes with PBS-T at room temperature, detection was carried out using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and blots were exposed to X-ray film to visualize the signals.

Tissue microarray construction and sectioning

MUC1 expression was assessed on microarrays containing 278 tissue sections from 110 human patients (tissue specimens were obtained from Dr Mahul Amin, Director, Pathology and Laboratory Support Core, Emory University School of Medicine, Atlanta, GA, USA with permission of the Emory IRB). A total of 66 formalin-fixed, paraffin-embedded radical prostatectomy specimens were selected for four different prostate tissue microarrays. Two tissue microarrays consisted of 66 primary prostate tumors and two tissue microarrays consisted of various kinds of non-neoplastic prostate specimens including normal prostate tissue and benign prostatic hyperplasia. Each array contained 100 sections, and included a section of normal lung as a positive tissue control for MUC1 expression. Tissue microarrays were constructed as follows: three tissue cores were chosen from each case with a diameter of 1 mm; a representative area of each 'donor' tissue block was punched using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA) and brought into a recipient paraffin block. Sections (5 μm) of the resulting tissue microarray block were cut and mounted to an adhesive-coated slide system (Fisher Scientific, Pittsburgh, PA, USA).

Immunohistochemistry

The slides were deparaffinized by three 3 min washes in citrus-based clearing solvent (Stephens Scientific, Riverdale, NJ, USA) at room temperature. Sections were rehydrated in a graded ethanol series (two 2 min washes each in 100, 95, 80, and 50% ethanol) at room temperature, followed by a 5 min flowing water rinse and a 20 min rinse in PBS. Rehydrated slides were quenched by two 10 min incubations in 50 mM NH_4Cl in PBS, and blocked for 30 min at room temperature in normal donkey serum at a final dilution of 1 : 50 in PBS. Slides then were incubated with a mouse monoclonal 214D4 primary antibody (hybridoma medium diluted 1 : 1 in PBS) for 1 h at 37°C. Slides were washed three times for 5 min each at room temperature in PBS, then incubated with FITC-conjugated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a final dilution of 1 : 10 in PBS for 40 min at 37°C, and washed three times for 5 min each at room temperature in PBS prior to mounting and visualization under a Zeiss Axioscope 2 fluorescent microscope equipped with a CCD camera. Exposure conditions were the same for all images taken at each magnification.

To verify the staining pattern observed with the 214D4 antibody, serial sections were stained with an affinity-purified, rabbit anti-human CT-1 MUC1 antibody specific for the cytoplasmic tail of MUC1 at a final concentration of 30 μg/ml in PBS (data not shown). MUC1 recognition by the CT-1 antibody ensured that potential differences in MUC1 glycosylation in the prostate cancer sections was not affecting the ability of the 214D4 antibody to recognize MUC1. In addition, this approach would detect forms of MUC1 lacking tandem repeat regions.[1,15] The use of CT-1 antibody on formalin-fixed paraffin-embedded tissue required antigen retrieval to optimize detection of MUC1. The antigen retrieval method detects a pattern of MUC1 expression in formalin-fixed paraffin-embedded tissue similar in intensity and distribution to that obtained on a frozen tissue (see Figure 4 for example). Therefore, in most cases, only the 214D4 patterns are presented herein since they are reflective of both antibody staining patterns. For antigen retrieval, slides were immersed in 250 ml of boiling sodium citrate buffer (0.01 M, pH 6.0) for 10 min, cooled at 4°C for 20–30 min, and then rinsed three times for 5 min each at room temperature in PBS. Antigen retrieval was performed after rehydration, but prior to quenching and blocking. The subsequent staining procedure was identical to that used for the 214D4 antibody. CT-1 was detected by FITC-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The combination of 214D4 and CT-1 antibodies was chosen to ensure that all forms of MUC1 potentially present in prostate tissues would be detected. Antibody 214D4 is not glycoform sensitive and detects all forms of MUC1 possessing the tandem repeat regions (MUC1/Rep and the secreted splice variant MUC1/Sec[1]). CT-1 recognizes all forms of MUC1 possessing the cytoplasmic tail (MUC1/Rep, MUC1/X, and MUC1/Y[1]).

Sections initially were scored positive or negative based on positive staining on any portion of the section. The positive staining then was scored according to its association with tumor or benign cells since both may be present within a section. The subcellular distribution of the staining pattern was determined for both positively stained tumor or benign cells as: apical only, diffuse cytoplasmic only, or apical and diffuse cytoplasmic (mixed) staining. Finally, a Gleason grade was determined for each tumor section (grading performed on serial sections stained by hematoxylin and eosin). Tumors were considered positive for MUC1 only when tumor cells displayed positive staining regardless of whether benign cells within the section were positive or negative. The threshold cutoff for positive staining was as follows: tumor tissue exhibiting only low-intensity diffuse cytoplasmic staining, barely detectable above background by 214D4 and negative by CT-1, was considered negative. If a section was missing at a position or damaged during processing in either the arrays evaluated for MUC1 staining or Gleason grade, it was not included in the final data set.

Results

Cytokine and DHT responsiveness of MUC1 in PrEC and prostate cancer cell lines

Western blotting was used to determine basal MUC1 protein expression in primary cultures of normal prostate epithelial cells and several human prostate cancer cell lines (Table 1, Figure 1). PrEC displayed low basal MUC1 expression; however, there was considerable heterogeneity among the prostate carcinoma cell lines. LNCaP, as well as the LNCaP sublines, C4-2 and C4-2B, had no detectable expression of MUC1, even with high protein concentrations (40-μg) and long exposure times. PC-3 (5-μg protein) had significant basal MUC1 protein expression, similar to the level observed in 40-μg of PrEC cell protein. DU-145 cells had the highest basal level of

Table 1 Human prostate cell lines evaluated

Cell line	Derivation	PSA positive	Androgen responsive	MUC-1 expression
PrEC	Normal prostate	ND	ND	Yes[a]
PC-3	Bone metastasis	No	No	Yes[a]
DU-145	Brain metastasis	No	No	Yes
LNCaP	Lymph node metastasis	Yes	Yes	No
C4-2	LNCaP subline	Yes	No	No
C4-2B	LNCaP subline	Yes	No	No

[a]Cytokine inducible.
ND, not determined.

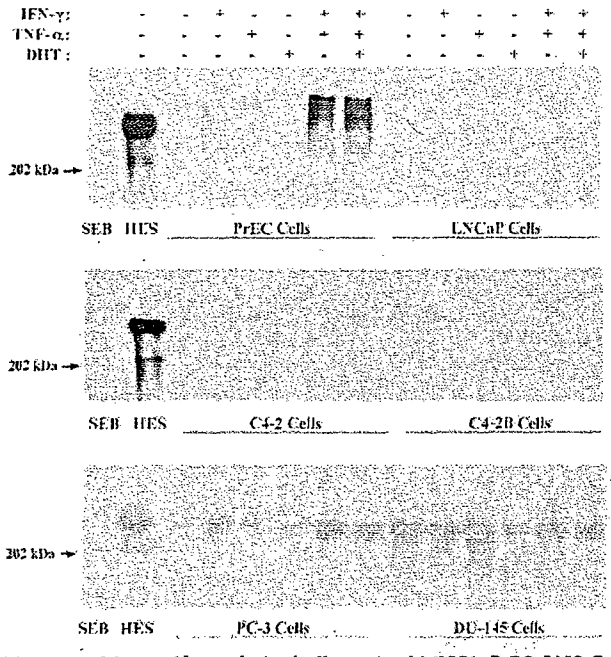

Figure 1 *Western blot analysis of cell associated MUC1. PrEC, LNCaP, C4-2, C4-2B, PC-3, and DU-145 cells were cultured in the presence or absence of the cytokines IFN-γ (50 ng/ml), TNF-α (100 ng/ml), and/or the androgen DHT (1 μM) as described in 'Materials and methods'. Following 24 h of serum starvation, cells were treated in serum-free medium for 24 h. Total cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and evaluated for MUC1 protein levels using the 214D4 antibody as described in 'Materials and methods'. HES cell protein was included as a positive control and sample extraction buffer (SEB) was included as a background (negative) control.*

MUC1 expression, equivalent to the level expressed in the positive control HES cell line.

In light of studies indicating that MUC1 expression is stimulated by steroid hormones and cytokines in other systems,[18,25,26] we evaluated the ability of IFN-γ, TNF-α, and DHT to induce MUC1 expression in PrEC and prostate cancer cell lines. PrEC expressed moderately higher amounts of MUC1 after treatment with IFN-γ or TNF-α alone; however, combined cytokine treatment produced a highly synergistic increase in MUC1 expression. DHT had no effect on MUC1 expression whether added alone or in combination with cytokines. None of the agents alone or in combination induced MUC1 expression in LNCaP, C4-2, or C4-2B cells. Since the levels of cytokines and DHT used in these studies were at the highest extreme of physiologically observed concentrations, we concluded that they had no effect on MUC1 expression in the LnCaP cell line or its derivatives. PC-3 cells showed a similar responsiveness as PrEC, although IFN-γ alone had a greater affect in the PC-3 cells. DU-145 cells, with the highest basal MUC1 expression, showed no further increase in response to cytokine or DHT treatment in any combination.

Immunohistochemistry of human prostate tissue sections

Immunohistochemical analysis of MUC1 expression was conducted on a tissue microarray containing 278 human prostate sections obtained from 110 individuals. Of the 278 sections evaluated, 103 (37%) were nontumor tissue (ie, non-neoplastic) and 175 (63%) contained tumor tissue (Figure 2). Of those 278 sections, 42 (41%) of the nontumor sections and 30 (17%) of the tumor sections stained positively for MUC1, suggesting that there is a loss of MUC1 core protein expression in prostatic tumors when compared with non-neoplastic prostate tissue. Epithelia in sections of normal lung included in each array were uniformly positive for MUC1 with both antibodies used in these studies (data not shown). Since the staining patterns for both antibodies were similar in intensity and distribution in a given section, images obtained using only one of these antibodies (214D4) generally are shown for simplicity. Figure 4 panels a and b show a representative comparison of staining patterns obtained with the two antibodies. Non-neoplastic tissues consistently displayed an apical staining pattern. While no attempt was made to characterize the incidence of staining within each section, that is, the percentage of cells staining positively for MUC1, it was evident that there was considerable variability in the incidence, intensity, and the subcellular distribution both between and within sections (Figure 3). Some sections showed an apical expression pattern while other sections showed

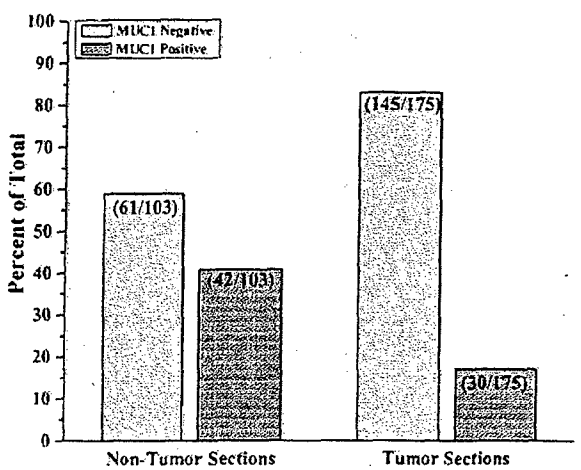

Figure 2 *Frequency of MUC1 expression in human prostate tissues. Tissue microarrays were stained with antibodies and scored as described in 'Materials and methods'. Lighter bars indicate MUC1-negative sections, while darker bars reflect MUC1-positive sections. The numbers in parentheses in each bar indicate the number of sections scored as positive or negative relative to the total number of sections surveyed in that category.*

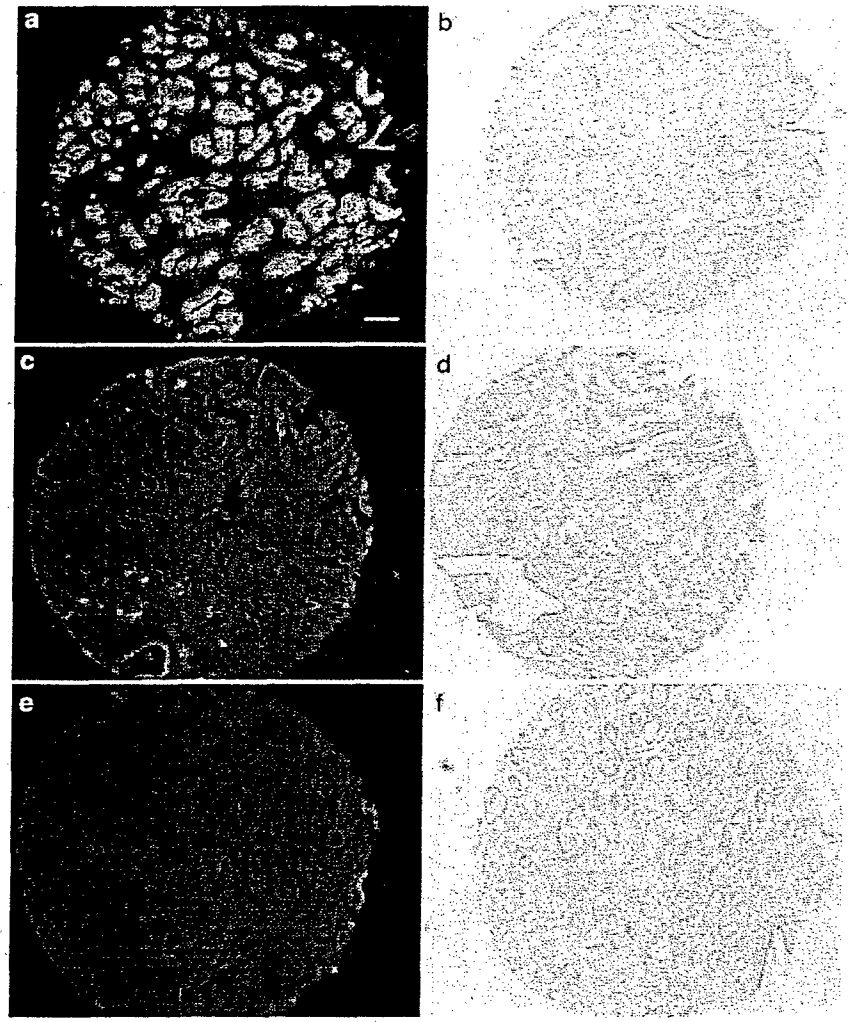

Figure 3 *Variable frequency and intensity of MUC1 expression in tumor tissue. Frequency of MUC1 detected in tumor cells by 214D4 ranged from ⩾90% (panel a, Gleason grade 3) of acini with all cells within acini staining positive with high intensity to ⩽1% (panel e, Gleason grade 3) of acini positive with ⩾99% negative (background). Intermediate frequency in panel c, Gleason grade 3, consisted of focal high intensity (⩽10% of acini combined with diffuse very low-intensity staining in ⩾90% of acini). Panels b, d and f show hematoxylin and eosin-stained sections from the same samples shown in panels a, c and e, respectively. Magnification bar located in panel a represents 100 μm and is equal for all panels.*

diffuse cytoplasmic staining or a mixture of both. Figure 4 illustrates the range of variability in MUC1 staining intensity and subcellular distribution that was observed, and the threshold for positive staining (panel f). Prostate intraepithelial neoplasia (PIN), considered to be a neoplastic precursor to malignant progression, displayed the same variability as tumor tissue (Figure 5).

The proportion of MUC1-positive sections that fell into each of the three Gleason grades examined (Gleason grades 3–5) was similar to that observed with the MUC1-negative sections (Table 2), indicating that there was no correlation between MUC1 expression and Gleason grade (MUC1-negative—52, 46, and 2% for Gleason grades 3, 4, and 5, respectively; MUC1-positive—63, 33, and 3% for Gleason grades 3, 4, and 5, respectively).

The Gleason grade for each positive tumor section also was correlated to the staining pattern in order to evaluate whether a loss of apical expression of MUC1 was associated with more progressive prostate cancers (Table 3). Of the Gleason grade 3 sections, 1/18 (6%), 5/18 (28%), and 12/18 (67%) displayed apical, diffuse cytoplasmic, or mixed staining, respectively. Of the Gleason grade 4 sections, 0/9 (0%), 4/9 (44%), and 5/9 (56%) displayed apical, diffuse cytoplasmic, or mixed staining, respectively. There was only one Gleason grade 5 section and it displayed a diffuse cytoplasmic staining pattern. Overall, most tumor cells displayed a diffuse cytoplasmic staining pattern regardless of grade. While these results suggest that a loss of restriction of cell surface MUC1 to the apical cell membrane, reflecting reduced cellular polarization, no Gleason grades 1–2 sections were available to evaluate this hypothesis further. Moreover, it was not clear that there had been a redistribution of MUC1 at the cell surface in higher grade tumor cells, since in many tumor cells, no distinction could be made between cell membrane-associated and cytoplasmic MUC1. Overall, no correlation was observed between higher Gleason grade and the subcellular distribution of MUC1.

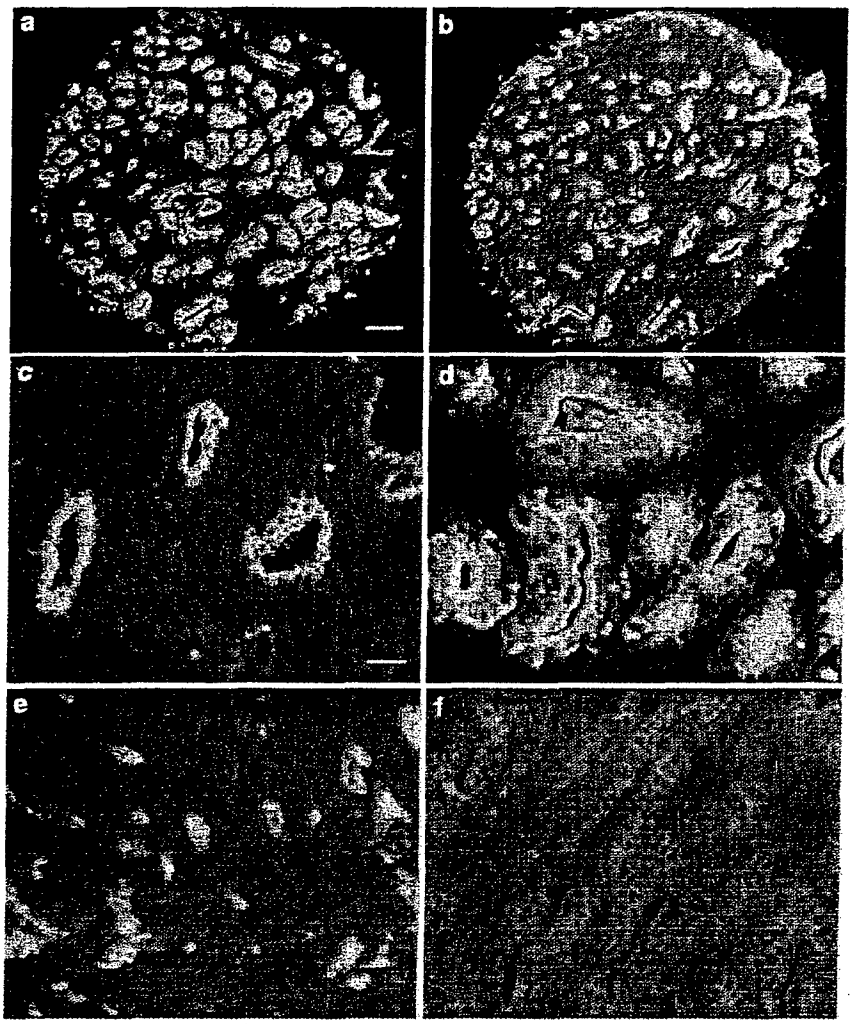

Figure 4 *Tissue and subcellular distribution of MUC1 epitopes recognized by antibodies 214D4 and CT-1. Serial sections of Gleason grade 3 (a and b) probed with antibodies 214D4 (panel a) or CT-1 (panel b) displayed a similar tissue distribution. Subcellular distribution of MUC1 recognized by 214D4 ranged from apical only (panel c, Gleason grade 3), apical and diffuse cytoplasmic (panels d and e, Gleason grades 3 and 4, respectively), to diffuse cytoplasmic-low intensity (panel f, Gleason grade 4). Magnification bar for panels a and b located in panel a is 100 μm, magnification for panels c–f located in panel c is 25 μm.*

Discussion

MUC1 expression in cancer progression and metastasis is characterized by increased levels, altered glycosylation, and aberrant surface distribution patterns.[2,4,8–12] In particular, the extracellular domain of MUC1 facilitates cancer progression. It also can promote adhesion due to the presentation of carbohydrate ligands that bind to selectin-like molecules on endothelial cells.[27] Finally, the large size of the extracellular domain presents a formidable barrier to immune cells, thereby contributing to decreased immune response (reviewed in Gendler[1]). Together, these properties of MUC1 enhance metastasis of MUC1-expressing cells (reviewed in Gendler,[1] Taylor-Papadimitriou *et al*[4]). These metastasis-promoting properties reside in the extracellular domain, and require cell surface expression to manifest. Intracellular MUC1 expression might still be able to alter cellular behavior by engaging signal transducing molecules via its cytoplasmic tail.[5–7]

In this study, basal MUC1 protein expression was evaluated in normal prostate epithelial cells (PrEC) and a series of metastasis-derived prostate cancer cell lines, and was consistent with one previous report.[28] LNCaP cells and sublines, representing a model for progression to androgen independence and skeletal metastasis,[29] were negative for MUC1 expression, suggesting that the lack of MUC1 expression is a characteristic of LNCaP cells passed onto the sublines. It should be noted that genetic changes known to occur in LNCaP and sublines did not include the locus for MUC1.[30] Interestingly, Mitchell *et al*[28] reported that the benign immortalized prostatic cell lines PNT-1A, PNT-1B, and BPH-1 all had detectable MUC1 protein levels. However, since PrEC failed to express significant MUC1 under basal conditions, and two metastasis-derived lines did express MUC1, it appears that lack of MUC1 expression does not correlate strictly with malignancy. Of the five prostate cancer cell lines evaluated, DU-145 cells had

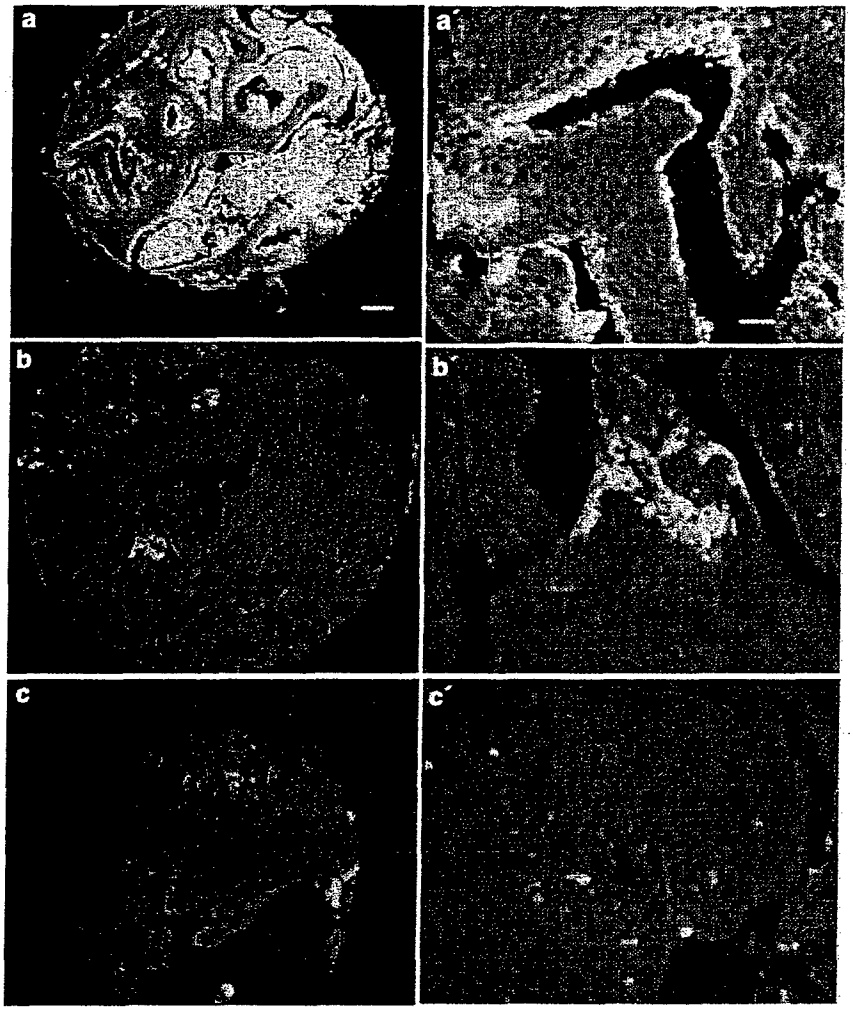

Figure 5 *Variable frequency, intensity, and subcellular distribution of MUC1 in PIN. Frequency of MUC1 detection by 214D4 in PIN varied from ⩾90% of acini positive (panel a) to ⩽10% of acini positive (panel c). Panels a′, b′ and c′ are higher magnifications of MUC1 positive areas seen in panels a, b and c, respectively. Within positive acini, frequency was uniform (panel a) or focal (panels b and c). Intensity varied from high-intensity apical staining on cells lining the lumen (panel a and a′), high-intensity cytoplasmic staining (panels b and b′) to low-intensity apical (panel b′) and cytoplasmic staining (nonluminal cells in panels a′ and c′). Magnification bar for panels a, b and c, located in panel a, is 100 μm; magnification bar for panels a′, b′ and c′, in panel a′, is 25 μm.*

Table 2 Distribution of MUC1-expression in prostate tumors by Gleason grade

	MUC1-negative tumor sections	MUC1-positive tumor sections
Gleason 3	76/95 (80%)	19/95 (20%)
Gleason 4	66/76 (87%)	10/76 (13%)
Gleason 5	3/4 (75%)	1/4 (25%)

Table 3 Staining pattern of MUC1-positive tumors

	Apical	Diffuse	Mixed
Gleason 3	1/18 (6%)	5/18 (28%)	12/18 (67%)
Gleason 4	0/9 (0%)	4/9 (44%)	5/9 (56%)
Gleason 5	0 (0%)	1/1 (100%)	0 (0%)

the highest basal expression of MUC1. Our results with PC-3 cells differ from that of Mitchell *et al*[28] who reported that unstimulated PC-3 cells were negative for MUC1 protein expression, whereas we found low basal MUC1 core protein expression in PC-3 cells. The most likely explanation for this discrepancy is that our use of MUC1 antibodies (214D4 and CT-1) not sensitive to glycoform variations, that is, more broadly recognizing MUC1, provides a more reliable method of revealing MUC1 protein expression.

In PrEC and PC-3 cells, MUC1 expression was elevated by cytokines. Androgen neither stimulated MUC1 directly nor potentiated MUC1 stimulation by cytokines in any cell line examined. Although this result was expected in androgen receptor-negative cell lines, the failure to see stimulation in the androgen-responsive LnCaP cell line may reflect an underlying defect in MUC1 expression since these cells also were unresponsive to cytokine stimulation. PrEC, in which androgen responsiveness may vary, uniformly responded to cytokine stimulation. Evangelou *et al*[31] reported androgen stimulation of MUC1 expression in PC-3 cells stably transfected with androgen receptor. Mitchell *et al*[32]

demonstrated stimulation of MUC1 expression by androgen and progesterone only in DU145 cells stably transfected with androgen receptor, while androgen receptor-positive LNCaP were unresponsive to both steroids. In contrast, DU145 and PC-3 exhibited increased cell surface MUC1 expression after prolonged exposure to 10 nM dexamethasone.[33] In the aforementioned studies,[31,33,34] increased MUC1 expression was assessed in terms of its detection at the cell surface and thus could represent a redistribution to the surface of MUC1 already present in the cytoplasm and/or production of an altered MUC1 glycoform detectable by the antibodies utilized rather than an increase in MUC1 core protein. Indeed, in the only *in vivo* model of hormonal escape in which MUC1 expression has been examined (beside LNCaP), MUC1 expression in the androgen-dependent parental PAC120 xenograft was reported to be focally cytoplasmic and either focally cytoplasmic or membrane associated in the hormonally independent derivatives.[35] Interestingly, these studies demonstrated that MUC1 was not coexpressed by the cells expressing secreted mucins whose elevated expression correlated with hormonal escape. Taken together, these observations suggest that MUC1 expression and subcellular distribution may be very sensitive to the cellular context and partially explain the variability observed in prostate tissue sections.

Available reports on MUC1 expression in prostate and during prostate cancer progression are contradictory in key respects. For example, in normal prostate epithelia, MUC1 expression varied from strongly positive immunoreactivity[8] to weak or no immunoreactivity.[9,12] Ho *et al*[8] reported intense staining in prostate adenocarcinomas ($n=10$). Zotter *et al*,[9] surveying several antibodies that produced weak or focally positive MUC1 staining in normal tissue, detected MUC1 in 9/10 tumors. However, they noted that a number of antibodies strongly positive on other tumors were weak on prostate cancers, indicating a difference in either the MUC1 glycoform itself or its relative abundance in prostate cancers. Utilizing antibody BC-1, recognizing hypoglycosylated MUC1, Ng *et al*[14] reported a positive correlation between increased MUC1 staining and progressive disease. Using antibody HMFG2 recognizing hypoglycosylated MUC1, Zhang *et al*[12] observed positive MUC1 staining (20% of cells) in 2/6 sections in normal prostate epithelia, 3/11 sections in primary prostatic tumors, and 5/9 sections in prostate cancer metastases. More recently, several groups have attempted to correlate MUC1 staining and cancer grade.[13,15,16] Kirschenbaum *et al*[13] reported a positive correlation between increased MUC1 staining and progressive disease in 34 patients, while Papadopoulus *et al*[16] showed no association with Gleason grade in 60 cases. Finally, Kirschenbaum *et al*[13] and Schut *et al*[15] reported that the expression pattern of MUC1 changed from an apical expression pattern to diffuse staining with more advanced prostate cancer while Papadopoulous[16] reported diffuse cytoplasmic and membrane staining even in low-grade tumors (Gleason 1–3). It is difficult to integrate this information into a cohesive picture of MUC1 expression during prostate cancer progression. While it seems that there is increased detectability of MUC1 associated with prostate cancer progression, it is not clear whether this reflects an increase in hypoglycosylated glycoforms, increased MUC1 core protein, or both. Two recent reports[15,36] illustrated the variability in MUC1 detection in prostate with different antibodies whose ability to recognize their MUC1 protein core epitope is variously affected by the degree of MUC1 glycosylation. These factors further complicate interpretation of previous studies.

The availability of tissue arrays presented us with the opportunity to perform the largest single study yet undertaken to examine MUC1 core protein expression in benign and malignant human prostate tissue. Applying a combination of antibodies that would detect all forms of MUC1,[15,37] we were able to detect MUC1 in only 41% of nontumor tissue (ie, benign), indicating that expression of MUC1 is variable in normal prostate. In contrast to studies utilizing antibodies recognizing hypoglycosylated forms of MUC1, our results demonstrated a reduction in MUC1 core protein expression in advanced prostate disease and no correlation with grade. These observations were confirmed in a gene profiling study recently published.[38] Positive MUC1 expression, although a strong predictor of tumor recurrence, was independent of tumor grade, and was present in only 26% of the 142 tumor tissues examined. Our data are consistent with that of Kirschenbaum *et al*[13] and Schut *et al*[15] in the sense that the expression pattern of MUC1 changed from apical to diffuse staining with more advanced prostate cancer, but it is not clear to what extent the MUC1 is cell membrane associated rather than cytoplasmic.

Another factor that may contribute to the variability in MUC1 expression in the prostate is the hormonal and cytokine mileau in patients from which the tissue samples are derived. As found for normal breast epithelia, breast cancer, and uterine cell lines,[18,26,39] we observed strong cytokine influences on MUC1 expression in normal prostate epithelia and certain prostate cancer cell lines (PC-3). In other prostate cancer cell lines, MUC1 either was constitutively expressed at high levels (DU-145) or not detected under any condition (LnCaP and derivative cell lines). In the latter case, we also failed to detect MUC1 expression in tumors formed when these cells were injected into the bone marrow of nude mice (JA Julian, DD Carson, and RA Sikes, unpublished results). Therefore, it is unlikely that our failure to detect MUC1 expression in these cells is due to a poor choice of cytokines as stimulants since bone marrow should provide a complex array of these factors and better mimic the environment of human bone metastases.

Overall, our data demonstrate that MUC1 expression is heterogeneous in both normal and malignant human prostate epithelia. Given its high degree of variable expression and sensitivity to environmental cues including steroid hormones and cytokines, we conclude that MUC1 expression is not likely to be a reliable indicator of prostate cancer grade or progression. Although altered MUC1 epitopes appear during the progression of prostate cancers, considering the incidence of MUC1 core protein expression in primary tumors, nonuniformity of MUC1 expression within tumors, and the reduction in membrane-associated MUC1 core protein observed in high-grade tumors, MUC1 also seems to be a poor choice as an immunotherapeutic target in prostate malignancies.

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